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14. **ABSTRACT**

We are developing an automated technique for scanning lymph nodes for small numbers of breast cancer cells using a mouse model. This technique involves the use of standard primary antibodies for tumor specific antigens and quantum-dot conjugates in place of chemical fluorophores combined with exhaustive confocal z-sectioning and computer analysis. We have verified that the quantum dot conjugates using antibodies to Brst and cytokeratin are functional in frozen sections from actual tissue but will have to do additional work to reliably identify cancer cells in an automated way. The primary problems to still be solved involve reducing antibody background. Once these have been solved, we expect to be able to test our automated approach for sensitivity against standard histological methods for detecting breast cancer cells in lymph nodes.

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INTRODUCTION:

This project is a Breast Cancer Concept Award in which the final goal is to test proof-of-principle whether an automated approach for detecting breast cancer cells in lymph nodes using quantum-dot-conjugated antibodies in conjunction with confocal microscopy and automated image analysis is feasible. Potential problems that must be solved are: 1) ability to visualize the quantum dot conjugates in cells or tissue using our confocal microscope, 2) whether quantum dot conjugated antibodies will penetrate as well into tissue sections as conventional antibodies (since the quantum dots are much larger than conventional fluorophores), 3) whether sufficient contrast is generated in confocal fluorescence images between stained cancer cells and unstained normal cells in the lymph node to permit the use of simple threshold-based image analysis techniques to identify cancer cells (if so, in principle an entire lymph node could be serial sectioned and examined exhaustively and automatically for a single tumor cell). and 4) whether exhaustive serial sectioning of lymph nodes and data acquisition is a tractable problem.
A major potential advantage of the quantum dot approach is their brightness and extreme resistance to photobleaching. This permits the use of very thick histological sections with most of the sectioning done optically and automatically by confocal. The bleaching resistance of the quantum dots means that avoiding repeated or prolonged laser exposure (a major limiting factor in z-sectioning with confocals) is not required. Given that the issues above are resolved in a satisfactory way, we intend to conduct a proof-of-principle test in mice using standard antibodies against tumor-specific antigens (currently cytokeratin and Brst2), comparing our techniques (which have potential for large-scale automation) to standard histological examination by a human pathologist. This was originally conceived as a one-year project. Unfortunately, Selma Dejgaard, one of the key personnel in this project, developed a serious back condition requiring surgery and has been on disability leave for most of 2004. This has slowed progress on this project and led to us requesting a no-cost extension of one year which was granted. We have nevertheless managed to test most of the preconditions for the success of the project (Task 1 on our SOW) as detailed below and to begin some of the elements of Task 2. We are confident we can complete the project successfully in the next 6 months to one year.

BODY:

We have used antibodies against two antigens found in breast cancer cells, cytokeratin and Brst2, which are used in pathology laboratories for screening lymph nodes for breast cancer cells. Our preference is to use in mouse lymph nodes, if possible, the same antibodies used for screening human tissue. Therefore, we have chosen antibodies for which cross-species activity in mice has been reported. We have initially used monoclonal antibodies (anti-cytokeratin, anti Brst2) and commercially-available biotinylated secondary antibodies to identify mouse breast cancer cells both in culture and in frozen sections from lymph nodes from a mouse model of breast cancer (both cancerous and control mice). Avidin conjugated to quantum dots (from Quantum Dots corporation) or to Cy3 was used, and images were taken with a Zeiss510 confocal and appropriate filter sets.

The anti-cytokeratin and Brst2 both gave strong staining of cancer cells in culture with the anti-cytokeratin staining intracellular fibers with a morphology resembling intermediate filaments, and the Brst2 giving cell surface staining. Staining was similar whether quantum dots or conventional avidin-Cy3 were used for visualization, in particular detailed intracellular patterns for cytokeratin staining were identical, and similar to published cytokeratin stains. The quantum dot signal was visualized without difficulty using the 488 nm Argon line and a bandpass emission filter centered on 600 nm. A test was conducted of photobleaching in which a full field of quantum-dot-stained cells was illuminated continuously with the laser at full intensity for one hour. There was no photobleaching of the quantum dots, rather intensity of emission climbed by about 30%. Similar results have been obtained elsewhere although the mechanism of this fluorescence increase is unknown.

An unanticipated problem found when frozen sections from lymph nodes were stained was cell surface staining of cells found in normal lymph nodes. This appears to be an unfortunate byproduct of the use of anti-mouse secondary antibodies which are probably staining mouse antibodies found on the surface of lymphocytes. We are currently investigating several solutions: 1) using primary antibodies from sources other than mice, 2) directly biotinylating the primaries to eliminate the need for the anti-mouse secondary, or 3)

blocking by adding a large amount of unlabeled anti-mouse secondary antibody prior to staining. Direct biotinylation of the primary is the most promising alternative as the biotinylation kits from Pierce are easy and reliable to use. We have gotten very good results with initial test biotinylations of proteins such as transferrin that we have in bulk. The biotinylated proteins can be added to cells and localized with quantum-dot avidin conjugates without difficulty. Based on these results we will begin direct biotinylation of antibodies and tests in tissue in the immediate future. Options 1 and 3 will be pursued if we have unexpected difficulty with direct biotinylation.

Despite the technical problems outlined above, we have been able to identify tumor cells in frozen sections of cancerous lymph nodes using the cytokeratin antibody. The artifactual background staining we found in normal cells is almost entirely confined to the cell surface giving the cell a ring or bubble-like appearance in a confocal section. As stated, this is probably due to staining of endogenous mouse antibody bound to the cell surface. In the cancerous lymph nodes there were large clusters of cells which contained fibers that could be stained with the cytokeratin antibody. These fibers were visible when the biotinylated secondary was visualized with either the conventional avidin-Cy3 or with the quantum-dot-conjugated avidin. This shows unequivocally that quantum dot conjugates can penetrate into the frozen section as well as conventional labels, and that there are no special technical problems with the staining caused by the use of quantum dots. The current images are unsuited for computer quantitation due to the high background, but as stated above, this is a problem we feel we can resolve.

We have also made progress in developing software that could be used to analyse confocal datasets. We have produced code written in C/C++ which first background corrects an image locally using a median filter with a large neighborhood size followed by thresholding the image. Confocal slices are processed one-at-a-time but the data are assembled at the end to identify above-threshold volumes that could be cancer cells or aggregates of cancer cells. More testing and tuning will be required on actual samples in order to maximize usability and fine-tune parameters like thresholds. Developing our own software allows us complete access to the source code and provides maximum flexibility for large-scale automation (which could be prototyped via Unix shell scripts) should that be a goal in the future.

KEY RESEARCH ACCOMPLISHMENTS:

● Immunofluorescence using quantum dots can successfully visualize tumor-specific antigens in breast cancer cells on our confocal and wide-field microscope systems.

● Quantum dot conjugates can penetrate frozen sections of lymph nodes in a satisfactory manner.

● We have confirmed extreme resistance of quantum dots to photobleaching in our hands.

● We have developed and tested computer code designed to find fluorescent objects (potentially tumor cells) in 3-D data sets.

REPORTABLE OUTCOMES:

It is anticipated that the project, when complete, will result in a publication. An abstract will be presented at the June, 2005 Era of Hope meeting in Philadelphia, PA.

CONCLUSIONS:

We have established that substituting quantum dots for conventional chemical fluorophores is feasible for antibody stains against breast cancer antigens in either mouse tissue culture cells or in lymph node sections. There are technical problems with high backgrounds in our stains of lymph nodes but they are not specific to quantum dots, and we think can be solved in the near future. We have developed and tested computer software that can be used to analyze 3-D data sets from lymph nodes once the background problem is solved. Once these problems are resolved, we plan to test our automated techniques for detecting cancer cells vs. standard histological techniques.

There is potential for automated techniques to show greater sensitivity than manual techniques to small numbers of cancer cells as conceivably an entire lymph node can be scanned and analyzed without human intervention after the initial sectioning. A more sensitive and accurate test for breast cancer metastasis should allow better tailoring of therapies, therefore saving lives.

REFERENCES:

n/a

APPENDICES:

n/a